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⑮ **Dye-providing composition, diagnostic test kit and their use in method for ligand determination using peroxidase labeled-receptor.**

⑯ A dye-providing composition comprises a water-soluble or -dispersible polymer, such as a vinylpyrrolidone polymer, and an imidazole leuco dye capable of providing a dye in the presence of hydrogen peroxide and a peroxidative substance. The weight ratio of polymer to leuco dye is from 10,000:1 to 100:1. The dye-providing composition can be included with a peroxidase substrate in a diagnostic test kit. A method for the determination of a ligand can be carried out using a peroxidase labeled-receptor for the ligand and the dye-providing composition described above. The method is particularly useful for the determination of human chorionic gonadotropin.

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Description

DYE-PROVIDING COMPOSITION, DIAGNOSTIC TEST KIT AND THEIR USE IN METHOD FOR LIGAND DETERMINATION USING PEROXIDASE LABELED-RECEPTOR

The present invention relates to a dye-providing composition comprising an imidazole leuco dye that provides a dye in the presence of hydrogen peroxide and a peroxidative substance, such as peroxidase. It also relates to a test kit including this composition and to a ligand-determining method using the dye-providing composition and a peroxidase-labeled receptor for the ligand.

There is a continuous need in medical practice, research and diagnostic procedures for rapid and accurate determinations of biological substances which are present in biological fluids at low concentrations. For example, the presence of drugs, narcotics, hormones, steroids, polypeptides, prostaglandins or infectious organisms in blood, urine, saliva, vaginal secretions, seminal fluids and other biological fluids has to be determined in an accurate and rapid fashion for suitable diagnosis or treatment.

To provide such determinations, various methods have been devised for isolating and identifying biological substances employing specific binding reactions between the substance to be detected (identified as a "ligand" herein) and receptors specifically reactive with that substance. Radioactive, fluorescent or enzyme labels have been used to detect the resulting reactive complex.

In recent years, the use of enzyme labels has received increasing attention because of various advantages over the use of radioactive and fluorescent labels. Assays using enzyme labels include what are known in the art as competitive enzyme immunoassays (EIA) and both direct and indirect enzyme linked immunosorbent assays (ELISA).

Another type of assay which has been developed is what is known in the art as an immunometric or a "sandwich" assay. Such an assay involves "sandwiching" the ligand (such as an antigen) with two or more receptor molecules (such as antibodies) which complex with the compound in a non-interfering manner and at different epitopic sites. In most sandwich assays, one or more of the receptor molecules are suitably immobilized on an insoluble carrier such as small particles, membranes, plates, or similar objects, and another receptor is suitably labeled, such as with an enzyme.

Peroxidase is one enzyme which has been used as a label in various assays, including specific binding assays of all types. Peroxidase acts on hydrogen peroxide as a substrate and can oxidize various chromogens or dye-providing materials to provide a detectable species in proportion to the amount of peroxidase present. Various dye-providing materials are known in the art, including benzidine and derivatives thereof.

In U.S. Patent 4,596,770, tetraalkylbenzidines are used in N-methylpyrrolidone as a dye-providing composition with peroxidase labeled antibodies in immunoassays. While such compositions may be useful if prepared and used immediately in a solution assay, it has been found that use of tetraalkylben-

zidines provide insufficient sensitivity in certain assays where the ligand is present in the test fluid in low concentrations. Also the benzidine compositions are relatively unstable. In addition, in certain assays carried out using filter membranes, the membranes must be pretreated because the dye obtained from tetraalkylbenzidines, such as tetramethylbenzidine, is water-soluble and would otherwise pass through the membrane inadvertently.

It would be useful to have a more stable dye-providing composition which can be packaged into kit form for lengthy storage. It would also be desired to have a more sensitive diagnostic method which could be sold for use in various environments, including doctors' offices and in a consumer's home.

The problems noted above are overcome with a dye-providing composition comprising a water-soluble or -dispersible polymer, the composition characterized wherein it comprises an imidazole leuco dye capable of providing a dye in the presence of hydrogen peroxide and a peroxidative substance, the weight ratio of polymer to leuco dye being from 10,000:1 to 100:1, and the polymer being a vinylpyrrolidone polymer, acrylamide polymer, acrylic or methacrylic acid polymer, polyethylene glycol or polyamine.

Also provided with this invention is a diagnostic test kit for the determination of an analyte as a result of the catalytic activity of peroxidase, the kit comprising:

- (a) a substrate for peroxidase, and
- (b) the dye-providing composition described above.

Further, a method for the determination of a ligand in an aqueous liquid comprises:

A. contacting a sample of the liquid with a peroxidase labeled-receptor for the ligand to form a reaction product of the ligand with the receptor,

B. prior to, simultaneously with or subsequently to contacting step (A), contacting the liquid sample with the dye-providing composition described above,

C. prior to, simultaneously with or subsequently to contacting step (B), separating the reaction product from unreacted materials, and

D. determining the presence or absence of the reaction product.

The present invention provides a greatly improved dye-providing composition that can be used to advantage where peroxidase or another peroxidative substance is involved in an assay. This dye-providing composition has improved stability because of the combination of a particular water-soluble or -dispersible polymer with an imidazole leuco dye which is oxidized to a dye in the presence of hydrogen peroxide and a peroxidative substance, such as peroxidase.

This dye-providing composition can be readily packaged into a diagnostic test kit which can be transported and stored for extended periods of time without significant loss in sensitivity. Very low levels of peroxidase labeled specific binding compounds can be detected with this invention because of the high sensitivity of the dye-providing composition.

In preferred embodiments where an assay is performed using a filter membrane, for example in a disposable test device, the membrane does not have to be pretreated in contrast to when tetramethylbenzidine is used as the dye-providing material. Surprisingly, the polymer in the composition of this invention facilitates keeping the imidazole leuco dye in solution in the dye-providing composition and facilitates immobilization of the resulting dye on the membrane during the assay.

The composition of the present invention is useful for providing a dye in the presence of a peroxidative substance, such as peroxidase, and hydrogen peroxide. The composition can be used in the determination of hydrogen peroxide or peroxidase, or any analyte which is capable of reacting, in one or more reactions, to produce hydrogen peroxide. For example, it can be used to advantage in assays for such analytes as glucose, galactose, amino acids, uric acid, triglycerides, creatine kinase (total or isoenzymes thereof), cholesterol and others known in the art where peroxidase is used in a sequence of reactions to produce a detectable species as a result of the presence of the analyte. Preferably, the composition of this invention is used in assays which involve specific binding reactions, such as immunoassays, as described in more detail below.

The composition includes one or more water-soluble or -dispersible polymers, such as vinyl pyrrolidone polymers, acrylamide polymers, acrylic and methacrylic acid polymers, polyethylene glycols and polyamines. These polymers can be either homo- or copolymers. Representative examples of useful polymers include, but are not limited to: poly(acrylic acid), poly(methacrylic acid), poly(acrylic acid-co-methyl acrylate) (90:10 weight ratio), poly(acrylamide), poly(acrylamide-co-acrylic acid) (50:50 weight ratio), polyamines such as those described in U.S. Patents 3,702,249 and 4,689,359. Particularly useful polymers are vinyl pyrrolidone polymers, that is homo- or copolymers prepared from vinylpyrrolidone such as poly(vinylpyrrolidone), poly(vinylpyrrolidone-co-acrylic acid) and poly(vinylpyrrolidone-co-acrylamide). These polymers facilitate in keeping the leuco dye in aqueous solution, as well as facilitating the immobilization of the resulting dye onto substrates, such as filter membranes, without the need to pretreat the membrane.

The polymers can also contain minor amounts (that is, less than 50 molar percent) of other ethylenically unsaturated polymerizable monomers which do not interfere with the function of the invention, or with the leuco dye.

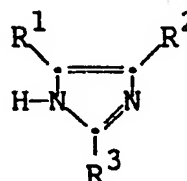
Poly(vinylpyrrolidone) is a preferred polymer.

The composition of this invention also includes one or more leuco dyes which are capable of providing a dye in the presence of hydrogen peroxide and a peroxidative substance. The result-

ing dye is generally detectable in the visible region of the electromagnetic spectrum (generally from 400 to 700 nm). Preferably, the dye is detected at from 500 to 650 nm.

Imidazole leuco dyes useful herein are either diarylimidazole or triarylimidazole leuco dyes. Many useful compounds are known in the art, including those described in U.S. Patent 4,089,747 and references noted therein, E.P. Publication 122,641 and Japanese Patent Publication 58(1983)-045,557.

The triarylimidazoles having the following general formula are particularly useful:



wherein R¹, R² and R³ are each an organic group such that at least one of them is an *ortho*- or *para*-hydroxy substituted aryl group of up to 18 carbon atoms, the other two groups being aryl groups chosen such that the imidazole oxidation potential is between -70 and +110 mV as measured by cyclic voltammetry against a standard calomel electrode using a carbon based electrode. Oxidation potential measurements can be made according to conventional electrochemical techniques (see, for example, Sawyer et al, *Experimental Electrochemistry for Chemists*, John Wiley & Sons, New York, 1974).

As used herein, the term "aryl" is meant to include aromatic hydrocarbon groups, such as phenyl, naphthyl or anthryl, tolyl, xylol and other substituted aromatic groups. The number of carbon atoms refers to the total number of nuclear carbon atoms as well as those in substituents. At least one of the R¹, R² and R³ groups has an *ortho* or *para* electron donating substituent such as an alkyloxy (-OR) wherein R is alkyl of 1 to 8 carbon atoms (for example, methyl, ethyl, isopropyl, t-butyl, hexyl, chloromethyl or methoxymethyl), or a dialkylamino wherein alkyl is as just defined. The R¹, R² and R³ groups can have one or more other substituents which are electronically compatible with the imidazole nucleus to provide a suitable dye upon oxidation. Further details of preferred triarylimidazole compounds and methods of preparing them are found in U.S. Patent 4,089,747.

Particularly useful triarylimidazole leuco dyes are: 2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole, 2-(3,5-dibromo-4-hydroxyphenyl)-4,5-diphenylimidazole, 2-(3-bromo-5-methoxy-4-hydroxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole, 4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxyphenyl)imidazole, 4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3-methoxyphenyl)imidazole,

2-(4-hydroxyphenyl)-4-(4-methoxyphenyl)imidazole, and
4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy)-3,5-dimethoxyphenylimidazole.

The amounts of leuco dye and polymer in the composition can be varied widely depending upon how the composition is used. Generally, the leuco dye is present in an amount of from 10^{-6} to 10^{-3} and preferably from 10^{-5} to 10^{-4} molar. The polymer amount depends upon the amount of leuco dye used. The weight ratio of polymer to leuco dye is from 10,000:1 to 100:1, and preferably from 5,000:1 to 500:1, in order to achieve the advantages described above.

The components of the composition described above are readily available commercially from a number of sources. Alternatively, they can be prepared using known starting materials and procedures, as described in U.S. Patent 4,089,747 and other references noted above.

The composition is generally in an aqueous medium although in one embodiment, minor amounts of water-miscible organic solvents can be included to solubilize the leuco dyes or other hydrophobic reagents. Methanol or other alcohols, acetone or acetonitrile are useful in this manner. Preferably, the leuco dye is dissolved directly with an aqueous solution of the polymer.

The compositions can also include other components as needed for a particular use. For example, the composition can also include either hydrogen peroxide or a peroxidative substance, but not both because the leuco dye would then be prematurely oxidized. Peroxidative substances are those capable of catalyzing the oxidation of another substance by means of hydrogen peroxide or other peroxides. Such substances include, but are not limited to, natural and synthetic peroxidases, cytochromes, hemin, forms of hemoglobin, alkaline hematin, iron sulfocyanate, iron tannate, chromic salts and the like. Peroxidase is a particularly useful peroxidative substance.

The composition can also include a specific binding compound which is attached to a peroxidative substance, such as a peroxidase-labeled immunologically reactive compound. A specific binding compound is a chemical or biological substance which will react specifically with another compound, such as avidin with biotin, or an antibody with its corresponding antigen. These materials are described in more detail below.

Electron transfer agents, that is compounds which facilitate the transfer of one or more electrons between compounds in oxidation-reduction reactions, can also be included in the composition. Many useful electron transfer agents are known in the art, such as phenazine methosulfate, benzo- and naphthoquinones as described in U.S. Patent 4,746,607. Particularly useful electron transfer agents are the phenols and anilines described in E.P. Publication No. 252,747.

A preferred composition of this invention is buffered to a pH of from 6 to 8 and comprises hydrogen peroxide, a phenolic electron transfer agent, poly(vinylpyrrolidone) and a triarylimidazole

leuco dye chosen from the list of preferred leuco dyes shown above with 2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole being most preferred.

A diagnostic test kit of this invention includes the composition described above as well as one or more other reagents, test devices or utensils for an assay. The kit includes a substrate for peroxidase, that is, a suitable peroxide, such as hydrogen peroxide.

Preferably, the kit also includes a test device for performing an assay, such as an immunoassay. Such a test device generally comprises a water-insoluble substrate having one or more test zones (such as test wells). The substrate is prepared from a water-insoluble material such as glass, polymeric materials, cellulosic materials and other materials known in the art. The device can be a test tube, petri dish, filter paper or test strip having the zones for reaction. It can also be a microtest plate having a multiplicity of preformed test wells.

The kit can also contain one or more of the following: a water-insoluble separation specific binding reagent which insolubilizes another compound by reaction specifically with it and separates it from other materials, peroxidase labeled-specific binding compounds (such as peroxidase labeled-antibodies), wash solutions, biotinylated antibodies, buffer solutions, reagent solutions, bottles, pipettes, test devices, prefilter devices, and other materials known to be useful in diagnostic kits to facilitate assays.

The present invention provides a method whereby a detectable complex between a ligand (a substance to be detected) and a receptor (a compound which reacts specifically with the ligand) is obtained. Advantageously, the method is simple and therefore can be performed in a doctor's office or in a consumer's home to provide immediate results. The test can be used to detect the presence or absence of a mono- or multivalent or multideterminant ligand in an aqueous liquid, such as a biological fluid. Preferably, it is used to detect a multideterminant ligand, such as hCG.

A monovalent ligand has a single epitopic site for complexation. A multivalent ligand has two or more epitopic sites for complexing with a multiplicity of the same specific binding receptor. A multideterminant ligand has two or more epitopic sites for complexing with a multiplicity of different receptors.

More specifically, the present invention can be used in the determination (qualitative or quantitative measurement) of a ligand in aqueous liquids to which there are naturally occurring or synthetically produced specific binding receptors. This determination can be made by merely determining the presence or absence of the ligand, or by quantitatively determining the amount of ligand. In particular, the invention can be used to assay biological fluids of animals, humans or plants, but preferably of humans. Such fluids include, but are not limited to, whole blood, plasma, sera, lymph, bile, urine, spinal fluid, seminal fluid, lacrimal fluid, vaginal secretions, sputum, perspiration and the like as well as stool specimens. It is also possible to assay fluid preparations of human or animal tissue such as

skeletal muscle, heart, kidney, lungs, brains, bone marrow, skin and the like.

The ligand of interest can be an immunological species, which is (1) any substance which, when presented to an immunocompetent host, will result in the production of a specific antibody capable of binding with that substance, or (2) the antibody so produced, which ligand participates in an antigen-antibody reaction. In some embodiments, avidin, biotin or an avidin or biotin derivative, or an enzyme or other label is suitably attached to the receptor molecule which reacts specifically with the ligand.

Representative ligands detectable with the present invention include primary amines, amino acids, peptides, polypeptides, proteins, lipoproteins, glycoproteins, drugs, haptens, enzymes, steroids, lipids, nucleic acids, hormones, vitamins, polysaccharides, glycolipids, alkaloids, organisms (bacteria, protozoa, fungi, viruses including retroviruses, rickettsia and the like) and components thereof, blood components, tissue and organ antigens and other materials known to one skilled in the art. In some instances, the ligand is an antibody which is directed against a drug, hormone, antibiotic or other compound having antigenic properties. Alternatively, the ligand can be an antigenic material. In still another embodiment, the immunological species is an antibody which is directed against another antibody (that is, an anti-antibody). Both monoclonal and polyclonal antibodies can be used, and they can be whole molecules or various fragments thereof. Preferably, monoclonal antibodies are used in the assays.

In a preferred embodiment, the method is useful for the detection of hCG as an early indicator of pregnancy. In this embodiment, one or more different antibodies to hCG are immobilized in the test device in order to provide reagents for forming a complex with hCG at different epitopic sites.

Generally, the method of this invention is carried out by contacting a peroxidase labeled-receptor for a ligand of interest with a sample of liquid suspected of containing the ligand in such a manner as to form a reaction product of any ligand present and the receptor in the test device. Generally, the liquid sample is applied to a test zone of a test device or placed in a test well, depending upon the configuration of the device. The presence or absence of the reaction product is then determined in a suitable manner after contact with the dye-providing composition of this invention and separation of the reaction product from unreacted materials. Separation can occur before, after or simultaneously with dye-formation.

The method of the invention can be a competitive binding immunoassay using both labeled and unlabeled receptor. Either bound (that is, complexed) or unbound (that is, uncomplexed) materials can be determined. Physical separation of bound and unbound materials, if desired, can be carried out using any suitable separation technique.

In a preferred embodiment, the method is what is known in the art as an immunometric assay. The details of such assays are provided in U.S. Patent 4,486,530. Such an assay can be used to determine

multivalent or bivalent determinants as described above, that is, having two or more epitopic sites for immunological reaction with two or more receptor molecules. In the sandwich assay, a second receptor is brought into contact with the ligand either prior to, simultaneously with or subsequent to contact of the ligand with the test device (and hence, contact of the ligand with the first receptor). The result is the formation of a complex of the two distinct receptors with the ligand. Preferably, at least one of the receptors is biotinylated. More preferably, the first receptor is biotinylated. The resulting complex is insolubilized when the avidin on an insoluble phase and biotin as part of the first receptor react, and the resulting insolubilized complex can be separated from unreacted material in the test device. One of the receptors or the insoluble phase can be labeled suitable for detection of the insolubilized complex.

In a preferred embodiment, a method for the determination of hCG in an aqueous liquid comprises the steps of:

A. contacting a sample of the liquid with a test device comprising a water-insoluble substrate having one or more test zones, and having in at least one of the test zones, a biotinylated antibody to hCG to form a reaction product of hCG with the biotinylated antibody at a first epitopic site,

B. prior to, simultaneously with or subsequent to the contacting step (A), contacting the liquid sample with a second antibody to hCG which is labeled and which reacts with hCG at a second epitopic site, to form a complex of hCG with the first and second antibodies,

C. contacting the complex with an insolubilizing separation specific reagent comprising an insoluble phase to which avidin is bound, to form an insolubilized complex through reaction of avidin with biotin,

D. prior to, simultaneously with or subsequent to the contacting step (C), contacting the complex with the dye-providing composition described herein while in the presence of hydrogen peroxide,

E. separating the resulting labeled, insolubilized complex from unreacted materials, and

F. determining the presence or absence of the labeled, insolubilized complex by measuring the amount of dye associated with the complex.

This method can be practiced in a doctor's office or at home for early determination of pregnancy by assaying urine samples.

The following examples are representative of the practice of this invention and is not intended to limit the scope of the invention.

Example 1: Dye-Providing Composition Useful for the Determination of Leutinizing Hormone

The following dye-providing composition is useful in the determination of LH in the practice of this invention.

A solution of 4,5-bis(4-methoxyphenyl)-2-(3,4-dimethoxy-4-hydroxy)imidazole leuco dye in methanol (15 mg/ml) was prepared. A sample of this solution (5 ml) was added to 500 ml of a solution comprising

sodium dihydrogen phosphate (100 mmolar, pH 7), polyvinyl pyrrolidone (1%), diethylenetriaminepentaacetic acid chelating agent (5 mmolar), hydrogen peroxide (5 mmolar) and 4'-hydroxyacetanilide electron transfer agent (5 mmolar) which had been adjusted to pH 7 using sodium hydroxide.

Example 2: Dye-Providing Composition Useful for the Determination of hCG

The following composition was prepared and used for the determination of hCG according to the practice of the present invention.

A first aqueous solution was prepared with sodium dihydrogen phosphate (10 mmolar), diethylenetriaminepentaacetic acid chelating agent (10 μ molar) and 4'-hydroxyacetanilide electron transfer agent (5 mmolar). This solution was adjusted to pH 6.8 with 10 molar sodium hydroxide.

A second aqueous solution was prepared with 20% (w/w) polyvinylpyrrolidone and 0.01% of the leuco dye of Example 1.

One part by weight of the second solution was added to nineteen parts of the first solution. Hydrogen peroxide (30%, 9.7 molar) was added to produce a final concentration of 10 mmolar hydrogen peroxide.

Example 3: Assay of Urine for hCG

Preparation of Insolubilizing Reagent:

The three solutions outlined below were continuously added to a 1365 ml vessel containing deoxygenated water at 80°C at the indicated rates:

Solution 1: Styrene (739 g), m & p-(2-chloroethylsulfonylmethyl)styrene (82 g) and 1-dodecanethiol (8.2 g) at 2.5 g/min. for 380 minutes.

Solution 2: Ammonium persulfate (19.7 g) and distilled, deoxygenated water (1152 g) at 2.14 g/min. for 380 minutes.

Solution 3: Sodium pyrosulfite (9.9 g) and distilled water (1152 g) at 2.27 g/min. for 380 minutes.

After 380 minutes, the reaction was stopped, yielding about 1218 g of latex at 33.4% solids. The latex was dialyzed for 3 days to yield a latex having 27.3% solids and a pH of 5. This latex was diluted to 13.5% solids. NMR analysis confirmed a 96:4 molar ratio of styrene to the second monomer. The resulting latex particles had an average diameter of about 0.67 μ m as measured by transmission electron microscopy.

A sample (0.75 ml) of the latex described above was diluted to 20 ml with borate buffer (50 mmolar, pH 8.5) and avidin (5 mg) was subsequently added. The resulting suspension was agitated in an end-over-end fashion at 37°C for 18 hours, followed by centrifugation. The supernatant was discarded and the particles washed once with buffer by centrifugation and resuspended in 10 ml borate buffer. Biotin binding analysis (that is, titration with tritium labeled biotin) indicated that avidin had been covalently attached to the particles (7×10^{-6} molar binding sites per 0.3% bead suspension) to form a reagent of the present invention.

A test device was used to determine hCG in a urine sample in the following manner. This device contained three test wells each having a filter membrane consisting of a commercially available nylon membrane coated with succinylated casein (1.07 g/m²).

A negative control test well of the test device contained MOPS buffer (2 mg) in polyacrylamide binder (60 μ g). MOPS buffer is 3-(N-morpholine)propanesulfonic acid (pH 7.5). The test sample well contained biotinylated anti-hCG antibodies (3 μ g) immobilized therein, and MOPS buffer incorporated therein in a separate location. A positive control test well contained biotinylated anti-hCG antibodies (3 μ g), MOPS buffer (2 mg) in a separate location therein and hCG (400 ml.U.).

A urine specimen, prefiltered to remove impurities, and containing about 50 ml.U./ml of hCG was added to each well of the test devices, followed by addition of peroxidase-labeled anti-hCG antibodies (40 μ l of a 10^{-9} molar solution). After a one minute incubation, the insolubilizing reagent described above was added (40 μ l of 0.42% dispersion) to each well and the fluid was allowed to drain through the membrane in each well.

After washing, a leuco dye solution (40 μ l) described in Example 2 was then added to each test well. After two minutes, the color that was formed on the membrane in each well was measured using standard reflectance equipment and procedures, and converted to transmittance density using the Williams-Clapper transform. The amount of dye measured was an indication of the amount of hCG in the urine specimen tested.

Example 4: Assay for Leutinizing Hormone (LH) Immobilization of Avidin on Particles:

A solution (50 ml, 0.05 molar, pH 8.5) of borate buffer containing thiomersal (0.01%) was placed in a polypropylene centrifuge tube, and to it was added 6 ml of a solution of egg white avidin (6 mg) dissolved in 6 ml of deionized distilled water. The tube was then capped and shaken vigorously, followed by addition of 1.35 ml of a dispersion (15.5% solids) of poly[styrene-co-m & p-(2-chloroethylsulfonylmethyl)styrene] (95.5:4.5 molar ratio) beads (average size of 2.54 micrometers) and rotation end-over-end for 24 hours.

The polymer beads having avidin covalently attached thereto were washed with glycine buffer (0.1 molar, pH 8.5) containing 0.01% thiomersal, and then resuspended in glycine buffer (0.1 molar) containing 0.01% thiomersal to produce a dispersion containing an insoluble separation specific binding reagent (0.3% solids).

Assay for LH:

A test device having three test wells like that described in Example 3 having a 5 μ m nylon filter membrane in each well which had been pretreated with casein, was used in this assay. Also used in the assay were: a biotinylated antibody to LH (0.044 mg/ml in phosphate buffered saline solution) prepared similarly to the biotinylated antibody to hCG described above, a horseradish peroxidase labeled

antibody to LH (0.001 mg/ml in phosphate buffered saline solution containing 0.5% bovine serum albumin), and the reagent described above comprising avidin on beads (0.9% solids, pH 8.5).

Several urine samples were tested in this assay:

(a) a sample collected the 13th day of a woman's menstrual cycle containing 18 mIU LH/ml, and

(b) a sample collected the 14th day of the woman's menstrual cycle containing 64 mIU LH/ml.

Both of these samples were taken from the same person and the LH content was determined by an LH radioimmunoassay kit available from Diagnostic Products Corporation.

A mixture of urine sample (a) 200 ml, the peroxidase-labeled antibody (35 ml) and the biotinylated antibody (10 ml) was prepared and incubated at room temperature for two minutes. The insolubilized avidin reagent (40 ml) was then added to the mixture and incubation was continued for another five minutes. The mixture was then transferred to one of the test wells of the test device, and fluid and unreacted materials were drained away leaving insolubilized complex formed during incubation on the filter membrane. The remaining insolubilized product was then washed twice with phosphate buffered saline solution (125 ml) containing 0.1% TWEEN 20 surfactant, (a polyoxyethylene sorbitan monolaurate) filtered again and then contacted with the dye-providing composition of Example 1 (50 μ l).

Urine sample (b) was similarly assayed using a second test well of the test device.

In both test wells, a color was seen on the filter membrane within five minutes. The color in the first well was light pink in color whereas the color in the second well was bright red in color.

Example 5: ELISA Determination of Streptococcus A Antigen After Extraction with Citric Acid

An isolate of Streptococcus A (20 μ l), obtained from a local hospital, was treated to extract antigen for one minute at 25°C using a solution of sodium nitrite (8 molar, 120 μ l) and citric acid (1.2 molar, 10 μ l). The extraction solution was then neutralized using 120 μ l of a solution of 3-(n-morpholino)propanesulfonic acid buffer (2 molar) and ethylenediaminetetraacetic acid (25 mmolar, pH 7.5).

A nylon membrane, incorporated into a disposable device, was coated with succinylated casein (1.07 g/m²).

An IgG fraction from rabbit anti-serum to Streptococcus A (Bacto Streptococcus Antiserum, Group A, Lot #2672-50, from Difco Labs) was purified by precipitation with a 45% saturated ammonium sulfate solution followed by dialysis to remove the excess salt. The purified IgG fraction was then immobilized onto poly[styrene-co-m & p-(2-chloroethylsulfonylethyl)methylstyrene] beads (90:10 molar ratio) to give a dispersion of reagent (0.3% solids) in 0.05 molar glycine buffer (pH 8.5), containing 0.01% thimerosal as a preservative.

The purified IgG fraction was also conjugated to horseradish peroxidase using succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate by a

procedure similar to the method of Yoshitake et al (Eur. J. Biochem. 101, 395-399, 1979), then diluted to give a solution of the conjugate containing 9 μ g/ml of IgG in 0.1% casein.

The bead suspension (40 μ l) was added to the disposable device, followed by the antigen extract (40 μ l), 0.1% casein (120 μ l) and conjugate (20 μ l). The mixture was allowed to incubate on the membrane for 2 minutes at 25°C. The membrane was then washed with phosphate buffered saline solution (320 μ l) and a dye-providing composition comprising the leuco dye 2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole (0.005 weight percent), sodium phosphate (5 mmolar, pH 6.8), polyvinylpyrrolidone (1%), hydrogen peroxide (10 mmolar), 4'-hydroxyacetanilide (5 mmolar) and diethylenetriaminepentaacetic acid (10 mmolar) was added. After 3 minutes and a further washing, the dye on the membrane was read by reflectance. The readings were converted to D_T using the Williams-Clapper transform. Results are shown in the Table below as the averages of 2 or 3 tests for each level (colony forming units, CFU) of antigen.

TABLE

CFU Group A Streptococcus	D _T
3 x 10 ⁵	0.174
1.4 x 10 ⁵	0.149
6.4 x 10 ⁴	0.115
3.0 x 10 ⁴	0.101
1.4 x 10 ⁴	0.077
6.4 x 10 ³	0.047
No Cells (background)	0.044

Claims

1. A dye-providing composition comprising a water-soluble or -dispersible polymer, the composition characterized wherein it comprises an imidazole leuco dye capable of providing a dye in the presence of hydrogen peroxide and a peroxidative substance, the weight ratio of polymer to leuco dye being from 10,000:1 to 100:1, and the polymer being a vinylpyrrolidone polymer, acrylamide polymer, acrylic or methacrylic acid polymer, polyethylene glycol or a polyamine.

2. The composition as claimed in claim 1 further comprising an electron transfer agent.

3. The composition as claimed in either of claims 1 or 2 buffered to a pH of from 6 to 8.

4. The composition as claimed in any of claims 1 to 3 wherein the leuco dye is a triarylimidazole.

5. The composition as claimed in any of

claims 1 to 4 wherein the polymer is a vinyl pyrrolidone homo- or copolymer.

6. A diagnostic test kit for the determination of an analyte as a result of the catalytic activity of peroxidase, the kit comprising:

- (a) a substrate for peroxidase, and
- (b) the dye-providing composition as claimed in any of claims 1 to 5.

7. The kit as claimed in claim 6 further comprising a test device comprising a water-insoluble substrate having one or more test zones.

8. The kit as claimed in either of claims 6 or 7 further comprising a peroxidase labeled specific binding compound.

9. A method for the determination of a ligand in an aqueous liquid comprising:

- A. contacting a sample of the liquid with a peroxidase labeled-receptor for the li-

gand to form a reaction product of the ligand with the receptor,

B. prior to, simultaneously with or subsequently to contacting step (A), contacting the liquid sample with the dye-providing composition as claimed in any of claims 1 to 5,

C. prior to, simultaneously with or subsequently to contacting step (B), separating the reaction product from unreacted materials, and

D. determining the presence or absence of the reaction product.

10. The method as claimed in claim 9 for the determination of hCG in a urine sample wherein the peroxidase labeled-receptor is a peroxidase labeled-antibody to hCG.

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(54) **Dye-providing composition, diagnostic test kit and their use in method for ligand determination using peroxidase labeled-receptor.**

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(57) A dye-providing composition comprises a water-soluble or -dispersible polymer, such as a vinylpyrrolidone polymer, and an imidazole leuco dye capable of providing a dye in the presence of hydrogen peroxide and a peroxidative substance. The weight ratio of polymer to leuco dye is from 10,000:1 to 100:1. The dye-providing composition can be included with a peroxidase substrate in a diagnostic test kit. A method for the determination of a ligand can be carried out using a peroxidase labeled-receptor for the ligand and the dye-providing composition described above. The method is particularly useful

for the determination of human chorionic gonadotropin.



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Y	WO-A-8 502 018 (IMMUNO CONCEPTS INC.) * Abstract; claims 1,2,4 *	1-10	C 12 Q 1/28 G 01 N 33/52
D,Y	FR-A-2 361 654 (EASTMAN KODAK CO.) * Claims 1-4 * & US-A-4 089 747	1-10	G 01 N 33/58 G 01 N 33/76
A	US-A-4 283 491 (EASTMAN KODAK CO.)		
P,X	EP-A-0 256 562 (FUJI PHOTO FILM CO., LTD) * Page 5, lines 37-45,53-56; page 7, lines 34-45 *	1-10	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C 12 Q G 01 N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 18-07-1990	Examiner CARTAGENA Y ABELLA P.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	